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Optic nerve hypoplasia in the fetal alcohol syndrome: a mouse model

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ABSTRACT

Optic nerve hypoplasia is commonly observed in children affected by the fetal alcohol syndrome, and is believed to contribute to their poor visual acuity. We have used a 'binge' model of alcohol abuse in an attempt to recreate this hypoplasia in a mouse model. Pregnant female (C57BL/6 × CBA)F₁ mice were injected intraperitoneally with a single dose of a 25% solution of ethanol (v:w), either on d 11 or d 12 of gestation. Optic nerves were prepared for transmission electron microscopy from offspring at 3, 6, 9 and 15 wk of age (n = 64). A systematic random sampling technique was used to analyse both the cross-sectional areas of the optic nerves from semithin sections, and the numbers and cross-sectional areas of myelinated axons from thin sections. We found no significant differences either in the cross-sectional area or in the number of axons in the optic nerves between 3 and 9 wk from control and alcohol-treated groups. From 9 to 15 wk, alcohol-treated groups showed a loss of approximately 25% of myelinated axons (65931 ± 2806 – 49186 ± 3194 : mean number of axons \pm S.E.M., respectively). Over the same period the number of axons in control groups was relatively stable (62087 ± 2043 – 64703 ± 3607). This resulted in an optic nerve with statistically significantly fewer myelinated axons at 15 wk in the alcohol-treated group, and was reflected in a trend towards a smaller cross-sectional area of the optic nerve in alcohol-treated groups. Analysis of axon calibre distribution within the optic nerves suggested that axons of all sizes were lost between 9 and 15 wk in the alcohol-treated groups. We suggest a delayed trophic mechanism for this late axon loss, and that the term optic nerve atrophy may be a more accurate description of the process.

Key words: Mouse; fetal alcohol syndrome; optic nerve hypoplasia; neurotrophic factors.

INTRODUCTION

The fetal alcohol syndrome (FAS) is a constellation of abnormalities recorded in the children of alcoholic mothers, with a reported incidence of between 1.1 per 1000 (Abel, 1984), and 1 per 750 (Sulik et al. 1981) live births in the USA. A similar incidence has been recorded in the west coast of Scotland (Beattie et al. 1983). FAS has been characterised clinically as the possession of a typical set of dysmorphic facial features, associated with somatic and mental growth retardation (Jones et al. 1973, 1974). Up to 90% of affected children have eye defects, and over half of these have either unilateral or bilateral optic disc hypoplasia (Strömland, 1987). Ophthalmoscopic observation has indicated that the optic nerve-head of affected children is significantly smaller than that of a normal child (Strömland, 1987), and this optic nerve

hypoplasia has been reported to be associated with poor visual acuity and nystagmus in humans (Zeki et al. 1992). In cases of congenital optic nerve hypoplasia, it has been demonstrated that the decrease in area of the optic nerve-head is related to a loss of axons in the nerve, which is directly related to a concomitant loss of retinal ganglion cells (see Lambert et al. 1987, for review). There are, however, no similar postmortem studies of FAS children to enable analysis of retinal ganglion cell numbers to be made. It is likely, therefore, that not only is the optic disc the clearest and often only visible sign of alcohol having affected the nervous system (Pettigrew, 1986), but this is also a likely location for many of the defects resulting in visual disability.

It is unclear whether the primary defect responsible for the optic nerve hypoplasia lies in the retinal ganglion cells, forebrain target tissue or glial elements.

Other workers have succeeded in showing only subtle changes in the timing and final degree of myelination following alcoholic insult during pregnancy (Phillips et al. 1991, 1992; Kjellström & Conradi, 1992). A model of the permanent optic nerve hypoplasia seen in FAS children has not been developed to date.

We have used a 'binge' model of alcohol abuse in mice, based on that described by Cook et al. (1987). A single high dose of ethanol is given intraperitoneally at a discrete developmental stage, in an attempt to reproduce developmental anomalies arising through toxic insult. Days 11 and 12 of gestation were chosen for alcohol exposure, being the time at which both retinal ganglion cells and forebrain target tissues are undergoing cell division and the first retinal ganglion cell axons are growing into the optic stalk (Drager, 1985). Using light and electron microscope observations on the optic nerve, we have measured the size of the optic nerve, and the number and calibre of the myelinated axons present. We have found that a single dose of alcohol during gestation is sufficient to cause a significant late reduction in the number of myelinated axons in the optic nerve. We tentatively suggest that the term 'optic nerve atrophy' may be more appropriate to describe the process that occurs.

MATERIALS AND METHODS

Preparation of experimental animals

Superovulated (C57BL/6 × CBA)F₁ mice were mated overnight and checked for the presence of a vaginal plug the following morning, which was designated d 1. Pregnant females were injected intraperitoneally (i.p.) with a 25% solution of ethanol (v:w) at 0.015 ml/g body weight at either 16.00 h on d 11 or 10.00 h on d 12 of gestation. Control animals were injected with saline at the same times.

Offspring were taken at 3, 6, 9 and 15 wk of age, deeply anaesthetised with Avertin and perfused transcardially with buffered paraformaldehyde/glutaraldehyde fixative. Optic nerves were rapidly and carefully dissected out, making sure that no undue tension was involved. The nerves were then placed into fresh fixative overnight, before postfixation in 1% osmium tetroxide and embedding in Araldite. Thin and semithin (approximately 1 µm) sections were cut from the middle of each optic nerve.

Morphometric analysis

Three electron micrographs were taken from 1% lead citrate and uranyl acetate stained micrographs of thin

sections of each optic nerve using a Philips EM301 transmission electron microscope at ×800, printed at a final magnification of ×3000, and viewed on a computer monitor at approximately ×18000. This was more than adequate for the identification of small diameter myelinated axons. A Joyce Loebel Magiscan system (Applied Imaging) coupled with a systematic random sampling method (Mayhew, 1990) was used to estimate the number of myelinated axons present. Sections were oriented randomly on the grids and in the grid holder prior to photography. Individual sampling squares were then systematically chosen according to a grid superimposed on 3 equal and equidistant 20° sectors marked from the centre of a mosaic of the 3 transmission electron micrographs. 200–300 axon profiles per nerve were counted and their cross-sectional areas measured. Only those axons which could definitely be identified as myelinated were included in the sampling protocol. The cross-sectional areas of optic nerves were measured from 4 to 8 toluidine blue stained semithin sections. Sample axon counts could then be extrapolated to give counts for the entire nerve.

Measurement of blood alcohol

We carried out acute experiments to measure the blood alcohol level reached following an ethanol injection. A group of mice were injected i.p. with alcohol at T = 0. One pair of mice was killed by cervical dislocation at T = 5, 30 min and 1, 2, 4 and 6 h. Blood samples were taken rapidly by cardiac puncture and immediately analysed for alcohol content with an Alcolmeter (Lion laboratories; AE-D2) calibrated against a standard.

RESULTS

Acute blood alcohol levels

Acute measurements of blood alcohol levels were made in mice following an i.p. injection (Fig. 1). Within 15 min of alcohol administration, and for at least 2 h afterward, the mice were inactive and obviously intoxicated. Peak blood alcohol levels of 400–450 mg% were reached within 5 min of alcohol injection, and showed an exponential rate of clearance over the 6 h monitoring period. Blood alcohol was negligible at 6 h. By comparison, values of between 300 and 800 mg% have been reported as lethal in the human (Riley & Lochry, 1982). If the concentration or quantity of the dose was raised much above the

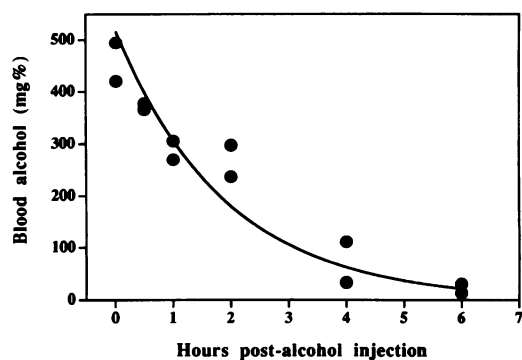


Fig. 1. Blood alcohol levels obtained via cardiac puncture following intraperitoneal injection of ethanol solution as described in the text. Each data point represents duplicate measurements made from a single female mouse at each of the postinjection times indicated. The line represents an exponential curve-fit for the data.

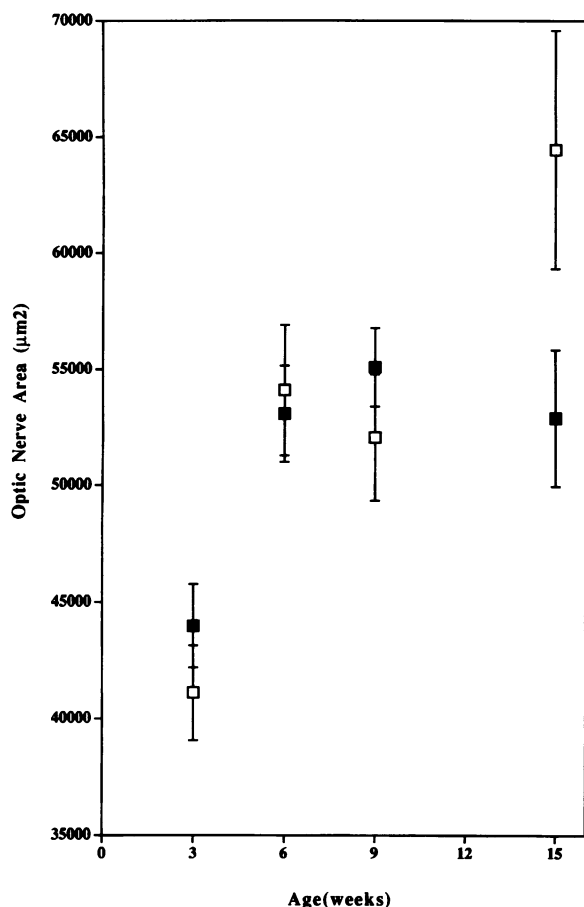


Fig. 2. Mean optic nerve areas calculated from 3–6 consecutive resin embedded sections, measuring the area of the nerve excluding the dural sleeve. Filled squares indicate results from alcohol-treated groups (data from d 11 and 12 alcohol treatments have been amalgamated as indicated in the text). Open squares represent control values ($n = 64$). None of the paired points was significantly different (Student's t test).

level used in the present study, a very high mortality of the female or the offspring was observed (data not shown).

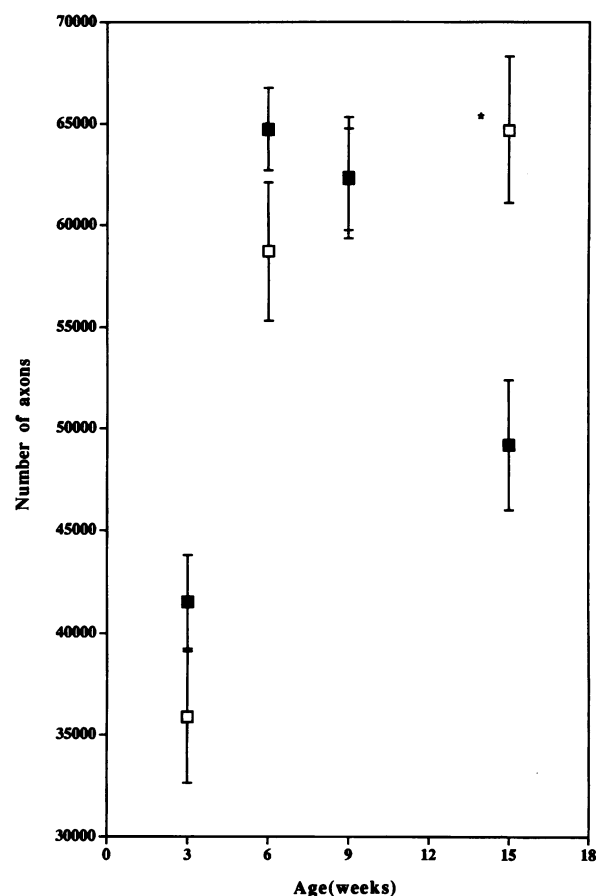


Fig. 3. Estimates for the number of myelinated axons present in each of the optic nerves assayed. The data from d 11 and 12 alcohol treatments have again been merged, and mean values from 3 to 6 individuals per point \pm S.E.M. are shown. Filled squares indicate results from alcohol-treated groups, and open squares represent control values ($n = 64$). Paired results were significantly different at 15 wk only ($P < 0.05$, Student's t test).

Optic nerve cross-sectional areas

The first question addressed was whether the ophthalmological observation of optic nerve-head hypoplasia could be reproduced in this model as a decrease in the cross-sectional area of the optic nerve in ethanol-treated mice. Cross-sectional areas (excluding the meningeal coverings) from the right optic nerve were measured from 3–7 consecutive sections per nerve. Results from offspring of pregnant females injected with alcohol on d 11 or 12 were not significantly different, and have been combined prior to presentation. Data from offspring of ethanol treated mice at 3, 6, 9 and 15 wk of age showing mean optic nerve area \pm S.E.M. are presented in Figure 2. First, control nerves increased linearly in size throughout the age range studied, from a mean cross-sectional area of $41113 \pm 2040 \mu\text{m}^2$ at 3 wk of age to $64438 \pm 5136 \mu\text{m}^2$ at 15 wk (mean \pm S.E.M.). Secondly, optic nerves in experimental mice were not signifi-

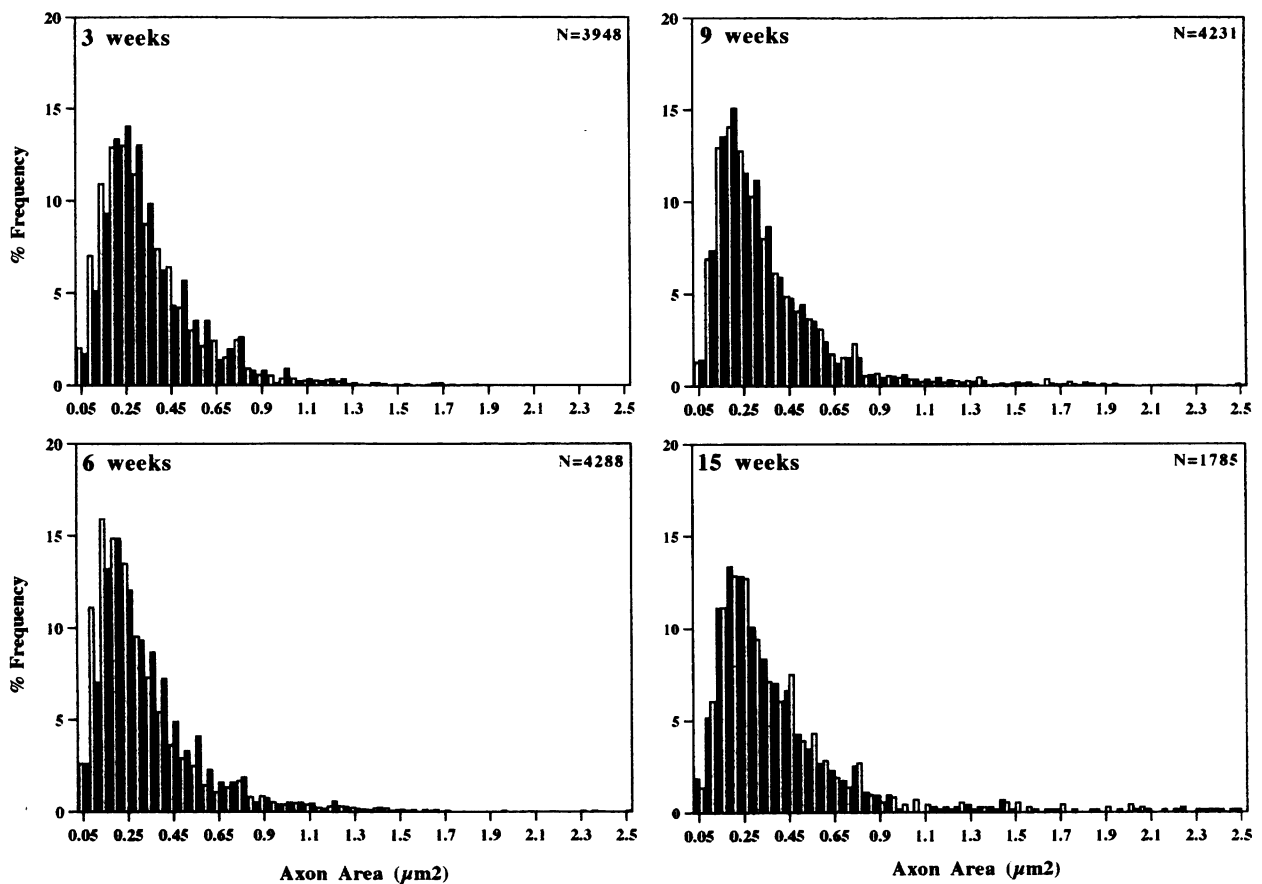


Fig. 4. Frequency histograms for the distribution of axon cross-sectional areas within the optic nerve. Data are represented as a percentage of the modal value at each age, which therefore becomes 100%. Results are shown for the axons counted at 3, 6, 9 and 15 wk and previously displayed in Figure 3. Filled bars are alcohol-treated groups and open bars are control groups. Histogram bins are 0.05 μm^2 .

ificantly different from control values between 3 and 9 wk $43990 \pm 1777 \mu\text{m}^2$ to $52833 \pm 2938 \mu\text{m}^2$ (mean \pm S.E.M.), respectively. However, at 15 wk the optic nerves of the experimental animals did not parallel the continued increase in cross-sectional area seen in the control animals, but showed an apparent decrease in cross-sectional area. This trend was not statistically significant ($P = 0.087$; t test).

Myelinated axon counts

The numbers of myelinated axons present were estimated using the sampling method described by Mayhew (1990) which relies on a proven systematic random sampling procedure (Parson et al. 1993, 1994). In the younger animals large numbers of unmyelinated axons were present, but by 9 wk very few remained. It should be remembered that during the early postnatal period up to 40% of the original number of retinal ganglion cells are lost (Potts et al. 1982). Estimates of total axon number for the nerves presented in Figure 2 are shown in Figure 3. Again, no

significant differences were found between the offspring of mice treated on d 11 or 12 of pregnancy, and results from these 2 groups have been combined. Numbers of myelinated fibres in control mice increased between 3 and 9 wk of age from 35868 ± 3229 to 62263 ± 2522 , respectively, but remained relatively constant to 15 wk 64686 ± 3596 (mean \pm S.E.M.). From 3 to 9 wk, alcohol-treated groups had slightly higher mean values, but were not statistically different from control groups, and paralleled the increase in numbers seen. Between 9 and 15 wk of age a marked decrease in the numbers of myelinated axons occurred, 62343 ± 2996 to 49187 ± 3194 (mean \pm S.E.M.), respectively. This loss of axons was significant ($P < 0.05$, t test), and resulted in a *hypoplastic* optic nerve in the adult with approximately 25% fewer axons than a control nerve at the same age (Parson et al. 1994).

This result indicates that the trend for the cross-sectional area of optic nerves from treated groups to decrease in size between 9 and 15 wk was underlain by a significant change in the number of axons which make up the nerve. The continued increase in the size of the optic nerve between 9 and 15 wk in control

animals was not accompanied by a concomitant increase in axon number, suggesting that this was due either to an increase in nonneuronal elements or to an increase in the areas of individual axons within the nerve. We therefore attempted to quantify the distribution of cross-sectional areas of the individual axons which make up the optic nerve.

Myelinated axon areas

The cross-sectional areas (excluding the myelin sheath) of all the axons sampled in the estimates of axon number were measured. These values were pooled for the total experimental (alcohol-treated) and control groups per age group, and binned histograms plotted for the distribution of axon areas (Fig. 4). In the histograms the modal value was taken as 100% and all other values were expressed as a fraction of this.

The histograms for results obtained at 3, 6, 9 and 15 wk showed a similar positively skewed form in each case. There was no significant variation in mean, median or modal values between ages or experimental groups. However, several points concerning the distributions deserve pointing out. First, at 3 and 6 wk there were more small axons present in the alcohol-treated groups when compared with the control groups. Secondly, the 'tail' of the distributions lengthened with increasing age, reflecting the greater number of large axons present. This was most apparent in the control groups, there being twice as many axons in the $> 1.8 \mu\text{m}^2$ bins in control than experimental groups at 15 wk. These 2 observations indicate that although neither the size of the optic nerve nor the number of axons present were significantly different at 3, 6 and 9 wk of age, there may be more subtle differences in the size profile of the axons which make up any particular nerve. Finally, the significant difference in axon number seen between 9 and 15 wk appeared to be reflected in a loss of all calibres of axons. Note that large axons had not been preferentially lost from alcohol-treated groups between 9 and 15 wk as they were not present in the alcohol-treated nerves at 9 wk. Instead, this reflected an increase in calibre of pre-existing axons between 9 and 15 wk in the control groups.

DISCUSSION

Previous reports of optic nerve hypoplasia have been made by ophthalmoscopic observation of the nerve-

head in affected children (Mosier et al. 1978; Strömland, 1987; Chan et al. 1991). We set out to produce a mouse model of optic nerve hypoplasia, and to determine if a reduction in axon number was responsible for the decrease in size of the optic nerve head observed.

The (C57BL/6 \times CBA)F₁ hybrid mice used in this study were chosen in preference to the C57BL/6J inbred strain of mouse which is known to have a genetic predisposition to congenital eye malformations and has previously been favoured in similar studies (Sulik et al. 1981) due to the poor breeding record of the C57BL/6J. However, it appears that F₁ offspring may show an increased prevalence of eye malformations compared with the inbred parent strain (Riley & Lochry, 1982). In the present study at least 3 mice in the control groups had unilateral failure to myelinate/demyelination of the optic nerve; also failure of the eyelids to open during the 2nd postnatal week has been seen.

Our experimental rationale results in a rapid rise in maternal blood alcohol levels following injection. Dilts (1970) has indicated that fetal blood alcohol levels generally equilibrate to within 15–30 mg % of the maternal level. Alcohol freely crosses the placenta (Kaufman & Woollam, 1981) and is cleared more slowly from the fetal than the maternal circulatory system (Cook et al. 1975). Wagner et al. (1970) have shown that the rate of elimination from the fetus is less than half that observed in the mother. Finally, the activity of alcohol dehydrogenase in the fetus is only 3–4% that of the adult (Riley & Lochry, 1982). Based on this information and our present results, we predict that fetal blood alcohol concentrations should reach values $> 400 \text{ mg \%}$, and remain in the circulatory system for up to 12 h. In the human this would be the equivalent of approximately a 4 d exposure.

Samorajski et al. (1986) demonstrated a slowing of unmyelinated axon loss and a lag in the onset of myelination in the optic nerve of rat offspring fed an alcohol-containing diet. Phillips et al. (1991) showed delayed rates of myelination following a full 3 trimester equivalent alcohol exposure regime. This is followed by 'catch up' growth and development, resulting in a relatively normal optic nerve, differing only in the possession of proportionately less myelin per axon. Further work from this group (Phillips et al. 1992) indicated that the alterations in rate and final degree of myelination are attributable to developmental changes in the oligodendrocyte population in the optic nerve. Similarly, hypomyelination and retardation of brain development (Hammer & Schiebel, 1981; Goodlett et al. 1989), and premature

onset and slowing of myelination have been reported following gestational alcohol exposure (Druse & Hofteig, 1977). In all these previous studies, the methods of alcohol administration limits the maximum blood alcohol level reached to between 150 and 200 mg %; this may be a significant factor in the difference in results obtained from this and previous studies.

The present study is the first to report a decrease in axon numbers in the mature optic nerve following a maternal alcohol insult. Interestingly, this has been obtained using an apparently less traumatic methodology, but one that achieves a higher blood alcohol concentration than those used by many other investigators.

Normally each retinal ganglion cell (RGC) has only a single axon (Potts et al. 1982). If this is the case, then the present result demonstrates a late decrease in number of RGC present, which can be relatively easily confirmed by anterograde labelling and counting of RGC. There is a loss of up to 40% of total RGC produced during normal development in rodents (Potts et al. 1982), but a stable number is generally reached by 6 wk postnatally. The late loss of axons shown in this study between 9 and 15 wk represents a phenomenon taking place subsequent to the normal period of cell death, and is not simply an augmentation of the existing process. Lambert et al. (1987) suggested a supranormal regression of RGCs rather than a primary failure to differentiate as the pathogenesis. We would concur that a primary failure to differentiate is not implicated, but that the late axon loss appears to be due to a late atrophy rather than hypoplasia.

We have previously described a small, but significant increase in the number of axons in the optic nerve of alcohol-treated mice at 9 wk (Parson et al. 1994). This was not maintained when sample size was increased in the present study. This may be a reflection of the ongoing process of myelination, which leads to a relatively large variation in the number of axons counted even in individuals at identical stages of development. In contrast, by 15 wk myelination is complete and counts show a smaller degree of variation.

Although not systematically investigated, little or no evidence of axonal degeneration or associated gliosis was seen in the 15 wk, alcohol-treated animals. This suggests that the clearing of debris is very rapid. Other researchers have specifically commented on the paucity of degenerating axon profiles seen during the development of the optic nerve, when the loss of axons is both profound and well documented (Perry et

al. 1983; Sefton & Lam, 1984; Crespo et al. 1985; Sefton et al. 1985).

According to 'trophic theory' (see Purves, 1988, for a review), neurons are dependent on limited supplies of trophic substances provided by their target tissues. Cells of the developing retina express receptors for the neurotrophins NGF, BDNF and NT3 (Maisonpierre et al. 1990), and retinal ganglion cells more specifically express both p140 and p75, high and low affinity NGF receptors (Zanellato et al. 1993). Retinal ganglion cells are dependent on their forebrain targets for survival (Vanselow et al. 1990). Carmignoto et al. (1989) have shown the rescue of retinal ganglion cells after optic nerve transection, following treatment with NGF, and Huxlin et al. (1993) have isolated a 480 kDa protein from the rat superior colliculus which appears to exhibit survival promoting activity for mouse retinal ganglion cells. Further, the production of NGF by the lateral geniculate nucleus early in postnatal development (Rickman et al. 1992), and BDNF by the superior colliculus throughout postnatal development (Friedman et al. 1991), have been demonstrated. It appears, then, that NGF and BDNF are likely candidates for a retinal trophic factor/s. Competition for a trophic substance limits the size of the innervating neuronal population. In the present study, a decrease in trophic substance production by the thalamus target tissue is implicated. This can either take the form of a reduction in production itself, or more normally a reduction in the size of the target and concomitant reduction in trophic factor production.

Voyvodic (1989) has shown that surgical alteration of target size leads to alteration not only in axon number, but also calibre and myelination in a peripheral nerve. In this case, an increase in target size is sufficient to cause normally unmyelinated axons to increase in diameter and become myelinated. The increased number of smaller myelinated axons present in alcohol-treated groups at 3 and 6 wk, and the fewer large axons at 15 wk, may be a subtle reflection of a similar process occurring in the central nervous system. But, more generally, this work demonstrates the control which target tissue exerts on its innervating neurons. Finally, retinal ganglion cells are also responsive to trophic factors produced intraretinally (de Araujo & Linden, 1993), although this has so far only been demonstrated at early developmental stages and may not be a significant factor in the later/mature stages studied here.

We would then propose a reduction in the size of the target tissue of the RGCs, namely the superior colliculus and lateral geniculate nucleus as the primary

cause of the reduction in axon number seen in the alcohol-treated animals at 15 wk of age. This is now being investigated using histological methods.

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